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Crystallization and preliminary crystallographic analysis of a plant type III polyketide synthase that produces benzalacetone

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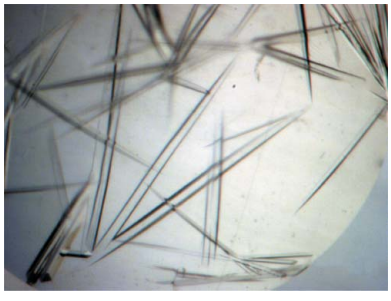

Benzalacetone synthase (BAS) from *Rheum palmatum* is a plant-specific type III polyketide synthase that catalyzes the one-step decarboxylative condensation of 4-coumaroyl-CoA with malonyl-CoA to produce the diketide 4-(4-hydroxyphenyl)-but-3-en-2-one. Recombinant BAS expressed in *Escherichia coli* was crystallized by the sitting-drop vapour-diffusion method. The crystals belong to space group $P2_1$, with unit-cell parameters $a = 54.6$, $b = 89.6$, $c = 81.1$ Å, $\alpha = \gamma = 90.0$, $\beta = 100.5^\circ$. Diffraction data were collected to 1.8 Å resolution using synchrotron radiation at BL24XU of SPring-8.

1. Introduction

Benzalacetone synthase (BAS; 384 amino acids; 42.2 kDa per monomer), a plant-specific chalcone synthase (CHS) superfamily enzyme of the type III polyketide synthases (PKSs), is thought to play a crucial role in the construction of the C_6–C_4 moiety of a variety of pharmaceutically important phenylbutanoids, including the anti-inflammatory glucoside lindleyin from the medicinal plant *Rheum palmatum* (Abe *et al.*, 2001). Although many of the type III PKSs catalyze iterative condensations of malonyl-CoA with a starter substrate, BAS catalyzes the one-step decarboxylative condensation with 4-coumaroyl-CoA to produce the diketide benzalacetone 4-(4-hydroxyphenyl)-but-3-en-2-one (Fig. 1*a*). The diketide-producing BAS obtained from *R. palmatum* shares ∼70% amino-acid sequence identity with the regular CHS (EC 2.3.1.74), which produces the tetraketide naringenin chalcone from 4-coumaroyl-CoA after three condensations with malonyl-CoA (Fig. 1*b*). Recent crystallographic and site-directed mutagenesis studies of several type III PKSs have begun to reveal that only a small modification of the active-site architecture generates the remarkable functional diversity of the CHS-superfamily enzymes (Ferrer *et al.*, 1999; Jez, Austin *et al.*, 2000; Austin *et al.*, 2004; Morita *et al.*, 2007). In *R. palmatum* BAS, the conserved active-site residues of CHS, Phe208 and Thr190 (corresponding to Phe215 and Thr197 in *Medicago sativa* CHS), are uniquely replaced by Leu and Cys, respectively. The conformationally flexible Phe208, which is located at the junction between the active-site cavity and the CoA-binding tunnel, is strictly conserved in all known type III PKSs and has been proposed to facilitate the decarboxylation of malonyl-CoA and to help orient substrates/intermediates during the sequential condensation reaction (Jez, Ferrer *et al.*, 2000). On the other hand, the chemically inert Thr190, which is altered in a number of divergent type III PKSs, has been shown to be important for steric modulation of the active-site cavity, thereby controlling the polyketide chain length and the product specificity (Jez, Austin *et al.*, 2000; Abe *et al.*, 2005). We previously demonstrated that replacement of Leu208 by Phe in *R. palmatum* BAS is responsible for the interruption of the polyketide-chain elongation at the diketide stage, while mutation of Cys190 to Thr did not significantly affect the diketide-forming activity of *R. palmatum* BAS (Fig. 1*b*; Abe *et al.*, 2003, 2007). Furthermore, our homology modelling studies suggested that unlike the case of CHS, *R. palmatum* BAS utilizes an alternative active-site pocket to lock the aromatic moiety of the coumaroyl starter substrate for the diketide-formation reaction (Abe *et al.*, 2007). To test this hypothesis and to further clarify the intimate

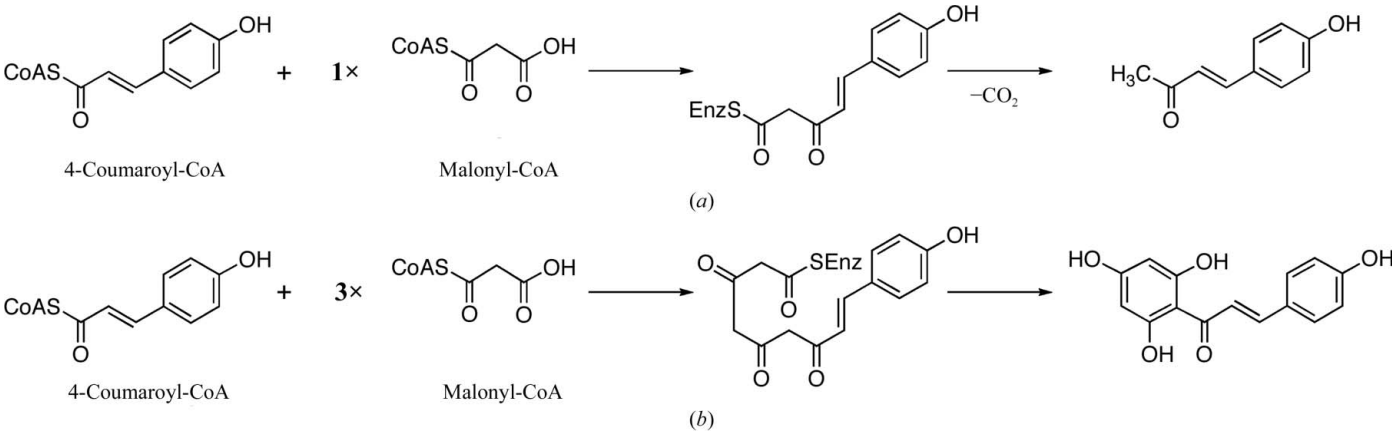

Figure 1
Proposed pathways for the formation of (*a*) 4-(4-hydroxyphenyl)-but-3-en-2-one by BAS and (*b*) naringenin chalcone by CHS.

three-dimensional structural details of the enzyme-catalyzed processes, we carried out a crystallographic analysis of *R. palmatum* BAS.

2. Experimental

2.1. Expression and purification

The pET22b(+) vector encoding full-length BAS (Abe *et al.*, 2001) was used as the template to amplify the BAS gene by PCR, using 5′-GCGCCCGGGAATGGCAACTGAGGAGATGAAGAAATT-GGC-3′ as the sense primer, which introduces a *Sma*I restriction site, and 5′-GCGGTCGACCTAGCTAATTACGGGCACACTGCGTA-GCA-3′ as the antisense primer, which introduces a *Sal*I restriction site. The amplified DNA fragment was digested with *Sma*I/*Sal*I and cloned into the *Sma*I/*Sal*I sites of a modified pQE81L expression vector (Qiagen) for expression as a glutathione *S*-transferase (GST) fusion protein at the N-terminus. Between GST and BAS, a Pre-Scission Protease (GE Healthcare) cleavage site (LEVLFQGP) was introduced. After confirmation of the sequence, the resultant expression plasmid was transformed into *Escherichia coli* M15. The cells harbouring the plasmid were cultured to an OD_{600} of 0.6 in LB medium containing 50 µg ml^{-1} carbenicillin at 303 K. Isopropyl β-D-1-thiogalactopyranoside was added to a final concentration of 0.4 m*M* to induce gene expression and the culture was incubated for a further 16 h at 303 K.

All of the following procedures were performed at 277 K. The *E. coli* cells were harvested by centrifugation at 5000*g* and resuspended in 50 m*M* Tris–HCl buffer pH 8.0 containing 0.2 *M* NaCl, 5%(*v*/*v*) glycerol and 2 m*M* DTT (buffer *A*). The cells were disrupted by sonication and the lysate was centrifuged at 12 000*g* for 30 min. The supernatant was loaded onto a Glutathione Sepharose 4B affinity column (GE Healthcare) equilibrated with buffer *A* and the column was then washed with buffer *A*. The GST tag was cleaved on the column by PreScission Protease overnight and the recombinant BAS was eluted with buffer *A*. The resultant BAS protein thus contains three additional residues (Gly-Pro-Gly) at the N-terminal flanking region derived from the PreScission Protease recognition sequence. After fivefold dilution of the protein solution with 50 m*M* Tris–HCl buffer pH 8.0 containing 5%(*v*/*v*) glycerol and 2 m*M* DTT, the protein solution was passed through a CIM-SO3-8 monolithic column (BIA Separations) and the flowthrough fraction was collected. The protein solution was further purified to homogeneity by chromatography on a Superdex 200HR (10/300GL) column (GE Healthcare) and was concentrated to 20 mg ml^{-1} in 20 m*M* HEPES–NaOH pH 7.5 buffer containing 100 m*M* NaCl and 2 m*M* DTT. The typical yield of protein was about 10 mg per litre of culture.

Dynamic light-scattering (DLS) analysis was carried out using a DynaProMSXTC molecular-sizing instrument (Protein Solutions Inc.). After centrifugation with a 0.22 µm Ultrafree-MC filter (Millipore) to remove particulate material from the protein solution, the solution properties of the purified protein were monitored. The data were acquired from 50 scattering measurements at 278 K; the data from five sets were analyzed using the *DYNAMICS* software package (Protein Solutions Inc.) and were averaged.

2.2. Crystallization and X-ray data collection

Initial crystallization attempts were carried out at 293 and 278 K using a 96-condition crystallization screen originally designed by the Mitsubishi Chemical Corporation with the sitting-drop vapour-diffusion method. Crystals were observed under many crystallization conditions and a crystallization condition containing 100 m*M* MES–NaOH buffer pH 6.5, 15%(*w*/*v*) PEG 8000 and 200 m*M* potassium thiocyanate was further optimized. Finally, diffraction-quality crystals were obtained at 293 K in 100 m*M* sodium citrate buffer pH 5.6 containing 16%(*w*/*v*) PEG 8000, 150 m*M* potassium thiocyanate using the sitting-drop vapour-diffusion method (Fig. 2). The optimized crystallization drops were prepared by mixing 0.5 µl protein solution and an equal volume of reservoir solution and were equilibrated against 100 µl reservoir solution. The crystals appeared

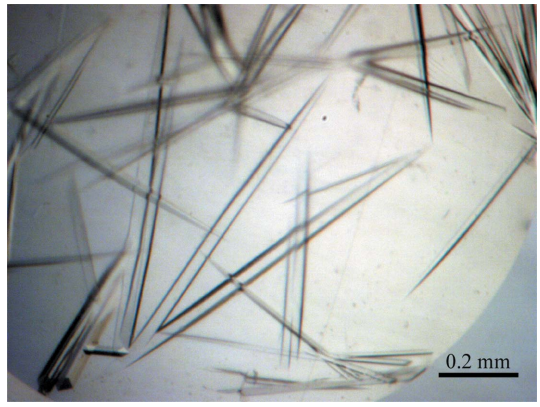

Figure 2
Crystals of BAS grown by the sitting-drop method.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P2_1$
Unit-cell parameters	
a (Å)	54.6
b (Å)	89.6
c (Å)	81.1
β (°)	100.5
Resolution (Å)	30.0–1.8 (1.86–1.80)
Unique reflections	69782
Redundancy	3.8 (3.8)
Completeness (%)	98.3 (97.3)
$\langle I/\sigma(I)\rangle$	25.4 (7.5)
$R_{\rm merge}$† (%)	9.6 (26.7)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of reflection hkl, $\sum_h$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection hkl.

reproducibly within 3 d and the largest crystal grew to dimensions of approximately 0.8 × 0.03 × 0.03 mm (Fig. 2).

A single crystal was divided into two halves of length 0.4 mm with a Micro-Scraper (Hampton Research) in order to fit into a 0.4 mm nylon loop. The crystal was transferred to reservoir solution containing 20%(*v*/*v*) glycerol as a cryoprotectant. After a few seconds, the crystal was picked up again in a nylon loop and flash-cooled at 100 K in a nitrogen-gas stream. X-ray diffraction data sets were collected on SPring-8 beamline BL24XU using a Rigaku R-AXIS V imaging-plate area detector. The wavelength of the synchrotron radiation was 0.82656 Å and the distance between the crystal and the detector was 350 mm. A total of 180 frames were recorded with 1° oscillation and 48 s exposure time. Data were indexed, integrated and scaled with the *HKL*-2000 program package (Otwinowski & Minor, 1997).

3. Results and discussion

The recombinant BAS was heterologously expressed in *E. coli* as a fusion protein with GST at the N-terminus. After cleavage of the GST tag, the purified enzyme migrated as a single band with a molecular weight of 43 kDa on SDS–PAGE, which agrees well with the calculated value of 42.4 kDa. In contrast, a gel-filtration experiment yielded a molecular weight of 83 kDa, suggesting that the recombinant BAS is a homodimeric enzyme, as in the case of other known type III PKSs (Austin & Noel, 2003). The DLS analysis after gel filtration revealed a monomodal distribution, with a polydispersity value of 14.0% and an estimated molecular weight of 81 kDa, which are in good agreement with the results of the gel-filtration analysis.

A complete data set was collected to 1.8 Å resolution. From the diffraction data collection, the space group was determined to be $P2_1$, with unit-cell parameters a = 54.6, b = 89.6, c = 81.1 Å, $\alpha = \gamma = 90.0$, $\beta = 100.5°$. Detailed data-processing statistics are shown in Table 1. With two monomers in the asymmetric unit, the Matthews volume ($V_{\rm M}$; Matthews, 1968) was calculated to be 2.3 Å^3 Da^{-1} and the estimated solvent content was 43.7%, which is in the range normally observed for protein crystals. Further structure determination using the molecular-replacement method, with the crystal structure of the *M. sativa* CHS (PDB code 1bq6; Ferrer *et al.*, 1999) as a search model, is in progress.

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